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Provisional Application Cover Sheet

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This is a request for filing a PROVISIONAL APPLICATION under 37 C.F.R. § 1.53(b)(2).

Docket Number: Q3465	Type a plus sign (+) inside this box	+

Inventor(s)/Applicant(s)			
Last Name	First Name	Middle Initial	Residence (City and either State or Foreign Country)
Gewirtz	Alan	M	Penn Valley, PA

Title of the Invention (280 Characters Maximum)

Design of Antisense Oligonucleotides and Short Interfering RNA (siRNA) Duplexes, Targeted to BCL 6 mRNA

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Enclosed Application Parts (*check all that apply*)

[X] Specification *Number of pages*: 16 [ ] Small Entity Statement

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Respectfully submitted,

Signature: _____ Date: 3/22/04

Typed or Printed Name: Alan Gewirtz

[ ] Additional inventors are being named on separately numbered sheets attached hereto.

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PROVISIONAL APPLICATION SUBMISSION TO USPTO – CONTENTS PAGE

Penn Docket Number	:	Q3465
First-named Inventor	:	Gewirtz
Submission Date	:	3/22/04
Prepared by	:	Matt Thomas

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Q3465/RBM

[473] Design of Antisense Oligonucleotides, and Short Interfering RNA (siRNA) Duplexes, Targeted to BCL 6 mRNA: Towards Rational Drug Development for Specific Lymphoma Subsets. Session Type: Oral Session

Joanna B. Opalinska, Anna Kalota, Alan M. Gewirtz Hematology, Pommeranian Medical University, Szczecin, Poland; Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

BCL 6 is a zinc finger protein, which acts as a sequence specific transcriptional repressor. Although BCL6 mRNA is ubiquitous, its expression is highest in germinal center B-cells where it is thought to repress the expression of genes involved in B-cell activation, cell cycle progression, and terminal differentiation. In non-Hodgkin lymphomas, Bcl-6 is the most frequently deregulated gene and abnormal expression is found in ~30-40% of Diffuse Large B-cell Lymphomas, DLBCL, and ~14% of Follicular Lymphomas, FL. Some have suggested that prognosis and BCL 6 expression are inversely related, while others suggest the opposite. Recently a subset of mutations within bcl-6 disrupting the negative autoregulatory circuit was described. These translocations were found in approximately 16% of DLBCLs, allowing us to hypothesize that bcl-6 might be an attractive therapeutic target in at least a subset of lymphoma patients. Accordingly, we sought to develop gene silencing antisense molecules targeted to bcl 6 mRNA. Our strategy was based on the use of self-quenching reporter molecules (SQRM) to rationally probe for hybridization accessible regions within a specific mRNA species. SQRMs are nucleic acid stem loop structures which emit a fluorescent signal after, and not before, hybridization to their mRNA target. To allow rational targeting of SQRM probes, a computer driven search algorithm was written and used to locate palindromic sequences ~5 bases in length separated by ~18 bases in the target mRNA sequence. Reverse complementary SQRM were synthesized to these sequences and used to probe a full length (2.4 kb), in vitro transcribed bcl 6 mRNA transcript. Of 19 SQRM evaluated, we found one (SQRM-1310) which consistently hybridized to its full length mRNA target (as revealed by a 10 fold increase in fluorescence compared to background). An in vitro RNaseH cleaving assay confirmed that SQRM-1310 could support specific mRNA cleavage while other sequences, though perfectly complementary, were ineffectual in this regard. We then demonstrated that SQRM-1310 could hybridize with its target mRNA in a solution containing total cellular RNA from a bcl-6 expressing cell line and that fluorescence developed was SQRM dose-dependent. We did not see any significant changes in fluorescence signal when the same molecule was incubated with RNA from K562 cells which do not express bcl 6. An antisense oligodeoxynucleotide corresponding to SQRM-1310, and other antisense predicted to be ineffective, were transfected into bcl 6 (+) Louckes Cells using an Amaxa nucleoporator. Cell viability was determined for 4 consecutive days. We found that cells transfected with Sequence1310 exhibited an ~50% drop in viability within 24 hours, while 3 other sequences were largely ineffective in this regard. Coincident with the drop in viability, we found a 7-fold decrease in bcl-6 mRNA expression in cells transfected with 1310, and little change in cells transfected with control oligonucleotides. Corroborating Western Blot data on bcl 6 expression were also obtained. Of particular interest, siRNA molecules targeted to the 1310 region failed to silence bcl 6 expression, though other regions were found sensitive to siRNA based silencing. The degree of silencing obtained using either approach was similar. We conclude that effective gene silencing oligonucleotides can be developed using our strategy of mRNA mapping and that those targeted to bcl 6 might find utility as novel agents for treating some patients with non-Hodgkin Lymphomas.

Design of Antisense Oligonucleotides and Short Interfering RNA Duplexes (siRNA) Targeted to BCL6 mRNA: Towards Rational Drug Development for Specific Lymphoma Subsets

Joanna B. Opalinska, Anna Kalota,
and Alan M. Gewirtz

Pommeranian Medical University, Szczecin, Poland and
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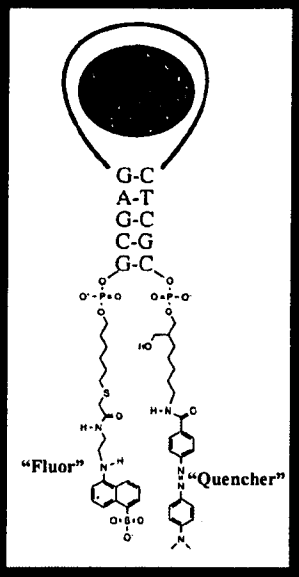
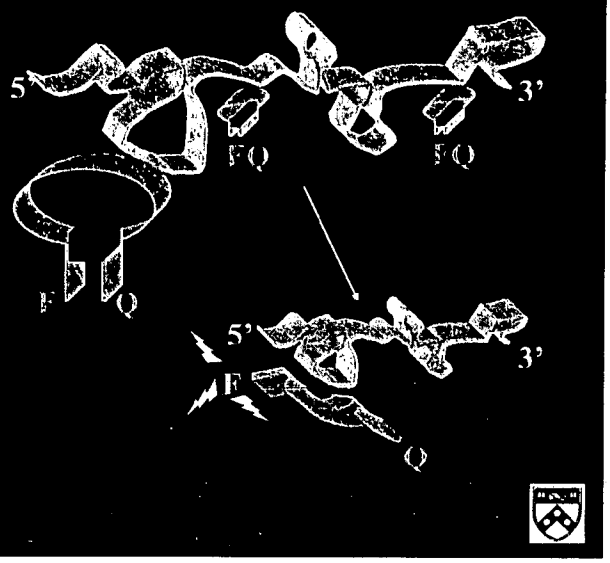
Bcl 6

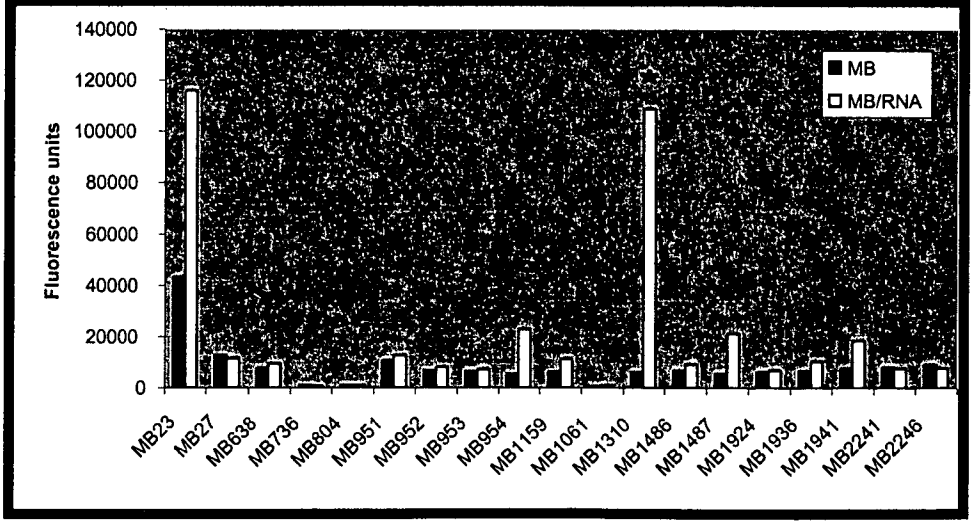
- Zinc finger protein- transcriptional repressor
- Inhibits expression of genes involved in:
  - B cell activation
  - Cell cycle progression
  - Terminal differentiation
- Frequently deregulated in lymphomas
- Potential therapeutic target in subset of lymphoma patients

Self-Quenching Reporter Molecules (SQRM)- aka "Molecular Beacons"

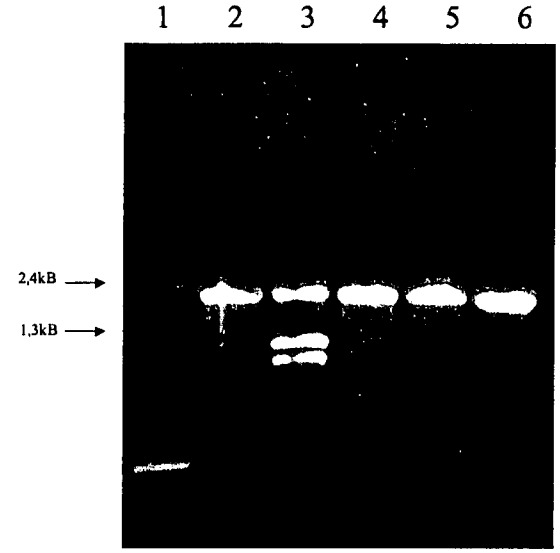




In Vitro Mapping of BCL6 mRNA

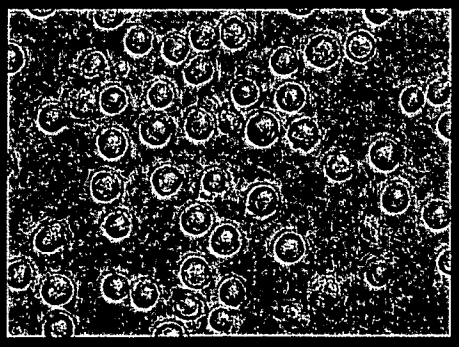


RNaseH Assay of SQRM #1310 and Control Sequences



1- RNA ladder
2-RNA/MB 951
3-RNA/MB 1310
4-RNA/MB 1487
5-RNA/RNaseH
6-RNA only

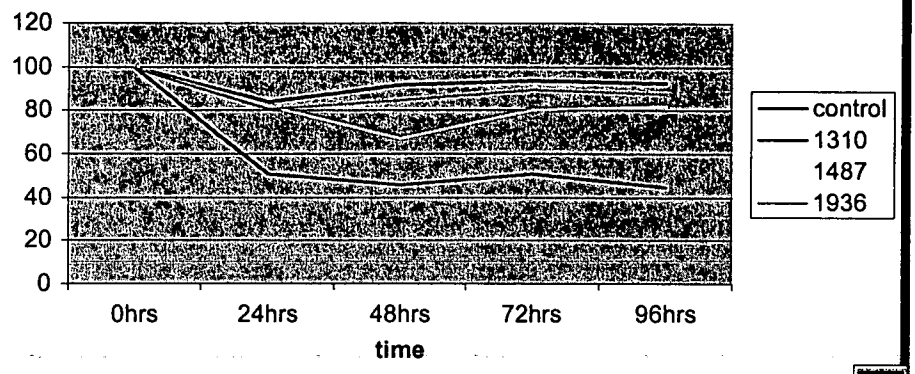
Nucleoporation of Louckes Cells With Fluorescein Labeled ODN



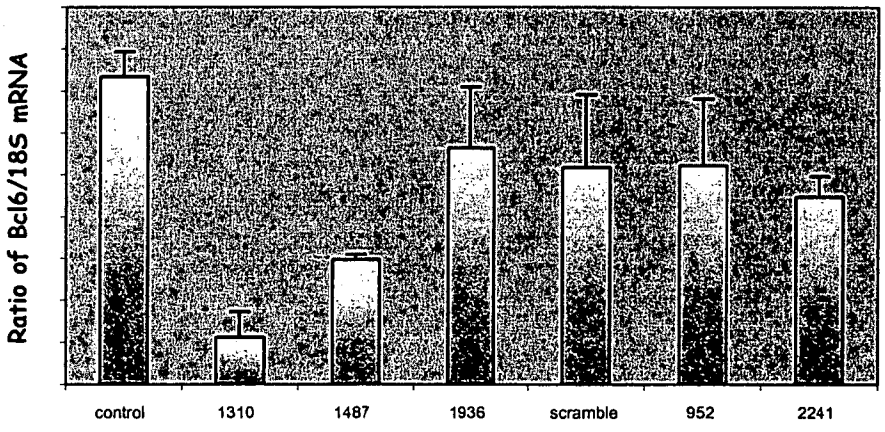
Phase

Fluorescence

Loucks Cell Viability Post Transfection with "Targeted" and "Random" Antisense Oligodeoxynucleotides

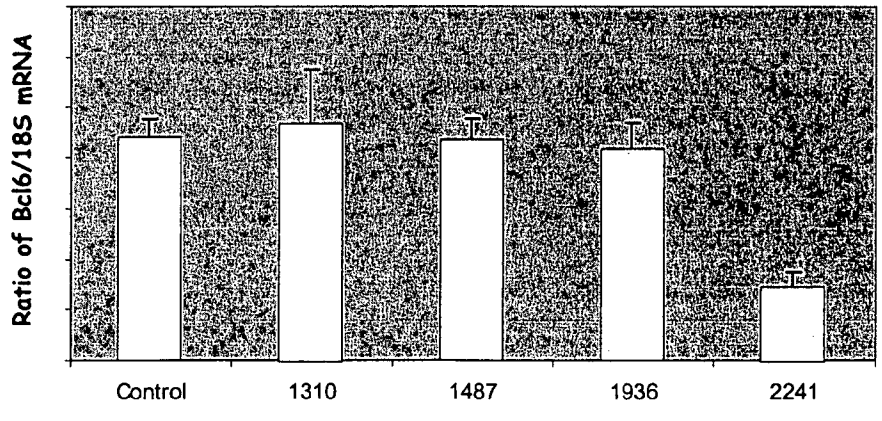


ODN Treated Loucks Cells-Effect on Bcl6 Expression

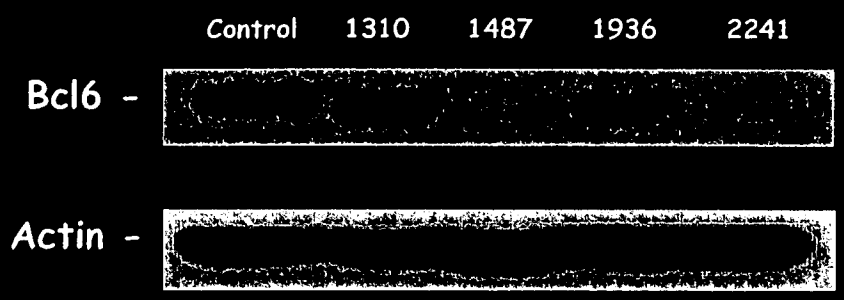


Bcl6 Western Blot in Loukes Cells Post Nucleoporation of Oligonucleotides

siRNA Treated Loukes Cells-Effect on Bcl6 Expression



Bcl6 Western Blot in Louckes Cells Post Nucleoporation of siRNA



Conclusions

- mRNA Structure- Important consideration for designing antisense nucleic acids
- Utility of SQRM Mapping for siRNA targets uncertain at this time
- Appropriately targeted DNA and siRNA molecules silence genes with equal effectiveness in vivo
- Bcl6 may represent a useful therapeutic target in a subset of lymphomas

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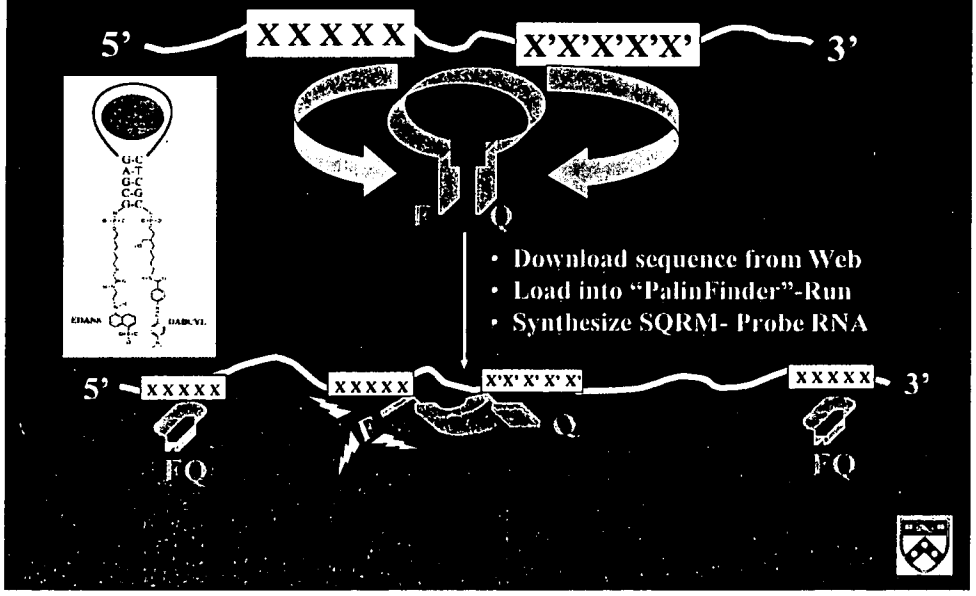
Palindrome Mapping Strategy



Summary

Targeting RNA for destruction is a widely employed strategy for gene silencing. Molecules utilized for this purpose are typically DNA, RNA or mixed nucleic acids (NA).that are complementary (antisense) or homologous to the mRNA of interset.
In mammalian cells targeting RNA with antisense NA has proved problematic. We have postulated that a significant issue underlying this difficulty is lack of ability to predict mRNA structure in vivo. Since silencing is ultimately effected by hybridization, and hybridization can only occur if the mRNA sequence to which the NA is targeted is accessible, it is clear that knowledge of structure may be enormously helpful in the design of these molecules.
Some more information???.....

Introduction

BCL 6 is a zinc finger protein, which acts as a sequence specific transcriptional repressor that binds to DNA with its amino-terminal POZ domain (Chang C.C., PNAS, 1996). Although BCL6 mRNA has been found in many tissues, the expression of the protein seems to be restricted to lymphocytes (Baron B. W.PNAS, 1993: Allman Blood, 1996) with the highest levels in germinal center B-cells. BCL 6 represses group of genes involved in B-cell activation and terminal differentiation (like *Blimp-1*), cell cycle control (*cyclin D2*, $p27^{KIP1}$), and inflammation (*MIP-1α, IP-10*)(Shaffer A.L, Immunity, 2000). It has also been postulated that BCL-6 indirectly induces c-myc (Shaffer, Immunity, 2000). The role of BCL-6 in apoptosis has not been well defined yet. There are reports of repressing genes involved in apoptosis by BCL-6, and thereby favoring neoplastic cell growth (with *PDCD2* as a target) (Baron B.W, PNAS 1993), as well, as activating apoptotic pathways by BCL-6 (with $BCL\text{-}X_L$ as a target) (Tang T.T JBC, 2002). Studies on knockout mice showed that BCL-6 is necessary for germinal center (GC) formation and T-cell dependent antibody responses. Mice lacking BCL-6 gene product fail to form GC and develop massive inflammatory response in many organs with eosinophilic infiltration and hyper IgE production (Ye B.H, Nat.Genet. 1997).
Bcl-6 is the most frequent deregulated gene in non-Hodgkin lymphomas (in ~30-40% of Diffuse Large B-cell Lymphomas, DLBCL, and ~14% of Follicular Lymphomas, FL) (Sanchez-Beato M, Blood, 2003; Shaffer A.L., Immunity, 2000), but we do not know precisely how the deregulated bcl-6 contributes to lymphomagenesis (Wang X, PNAS, 2002). The altered expression of Bcl-6 arises mostly from 3q27 translocations where the 5' non-coding region is replaced by heterologous promoter leading to over-expression of the protein (Harris N.L Blood, 1994; Ye B.H. EMBO 1995). The same region is also subject to somatic mutations in normal germinal center B cells and GC derived non-Hodgkin lymphomas (Artiga M.J. AmJ Pathol.2002). The level of *bcl-6* over-expression seems to depend on partner gene involved in the translocation. When *bcl-6* is translocated to an Ig locus the mRNA and protein levels are much higher then in translocations to a non-Ig locus. The prognostic value of *bcl-6* translocations/mutations in lymphoma patients has been a subject of controversy. Although BCL 6 over-expression seems to correlate with higher patient's age and LDH levels,(Jerkeman M. Int.J Oncol. 2002; Vitolo U, Leukemia, 2002) there are reports in the literature that bcl-6 overexpression predicts improved survival (Braaten K.M. Clin cancer Res. 2003; Vitolo U, Leukemia, 2002; Artiga MJ, AmJ Pathol.2002; Lossos IS, Blood 2001) but there are also studies demonstrating poor survival in these patients (Barrans SL, BrJ Hematol. 2002; Ueda C, Leukemia&Lymphoma 2002; Kawasaki C, Leuk&Lymphoma 2001; Chang CC, Arch Pathol Lab Med. 2003). In general, it seems that not the *bcl-6* translocation itself, but the partner in this translocation is an indicator of prognosis, because *non-Ig-bcl6*

transformations predict poor prognosis compared with *Ig-bcl6* (Akasaka T. Blood 2000). Recently Pasqualucci et al. described a subset of mutations within bcl-6 disrupting the negative autoregulatory circuit. These translocations were found in approximately 16% of DLBCLs devoid of chromosomal translocations, but they were not found in normal GC B cells.
Based on all these findings we have hypothesized that bcl-6 may be an attractive therapeutic target at least for a subset of lymphoma patients. We decided to identify a sequence within its mRNA, which would be accessible for gene silencing strategies.

Materials and methods

Cloning and in vitro transcription

An insert containing full length (2,4kB) bcl-6 coding sequence (kind gift of R. Dalla-Favera, Columbia University) was sub-cloned into pcDNA3 plasmid using KpnI and HindIII cloning sites, and then transcribed in vitro using T7 polymerase (Promega, **where from??).** Transcribed bcl-6 mRNA was used for subsequent experiments.

Self quenching reporter molecules (SQRM)

Potential SQRM sequences were identified by computer algorithm to locate palindromic sequences ~5-7 bases in length separated by intervening sequence, ~18-20 nucleotides in length. **Sequences are given in tab. 1** SQRM were synthesized by Nucleic Acid Facility at the University of Pennsylvania with Fluorescein at the 5' end and Dabcyl (as a quencher) at the 3' end. Sequences of these molecules are given in tab.1. In vitro transcribed bcl-6 mRNA was introduced into wells on a 384 plate and individual SQRM molecules were added to the wells in the presence of buffer (100mM Tris and 2 mM $MgCl_2$). Fluorescence was quantitated over time after 30 min incubation at 37°C in a plate reader and compared to an arbitrary 100% control, which was the exact complement of the SQRM probe.

Table (sequences-see at the end of the discussion)

RNase H cleaving assay

Diverse SQRM were tested in the presence of bcl-6 mRNA and RnaseH. For this purpose 1uM of SQRM was preincubated with 1uM mRNA in the presence of 100mM Tris and 2mM $MgCl_2$ at 37°C for 10 min to allow hybridization, Then RNaseH buffer and enzyme were added and the mixture was incubated for another 10 min at 37°C. The reaction was stopped by adding Proteinase K and the whole reaction mixture was resolved on 1% agarose gel.

SQRM testing with cellular RNA

We have chosen a bcl-6 expressing cell line Loucke, and non-expressing K562 cell line. The total RNA was isolated from the cell lines with Qiagen mini Kit, and the yield measured spectrofotometrically. A total amount of 20 000μg and 40 000μg of RNA isolated from these cell lines was incubated in the fluorimeter with the hybridizing 1310 molecule and compared to the background fluorescence, arbitrary 100% fluorescence, and fluorescence with 1μM in vitro transcribed RNA (IVT-RNA).

Cell culture and transfection with nucleic acids

Bcl-6 expressing human Burkitt lymphoma cell line Louckes **(kindly provided by your friend from Yale)** was maintained in RPMI 1640 media supplemented with 10% FBS (Hyclone) and 0,5% Penicilin/Streptomycin at 37°C, in fully humidified incubator (95% humididty) and 5% CO_2. The transfection procedure was performed with nucleoporator (Amaxa, Germany) according to the manufacturer's procedure. Briefly, on the day of transfection cells were washed twice in PBS and re-suspend in 100μL of nucleofection solution and nucleoporated in the presence of 5μg nucleic acid with the appropriate program. Immediately after the procedure $1x10^6$ cells were put into 1,5mL fresh media and cultured for another 6 hours in case of antisense ODN, and 48 hours in case of siRNA molecules.
The viability of cells was assessed with trypan blue exclusion method. Cells were checked for viability every 24 hours for 4 days.

Nucleic acid molecules
Antisense ODN were synthesized and HPLC purified by IDT **(California??).** At the 3'- and 5'- end of the molecule each 5 bonds were modified phosphorothioate, the rest was phosphodiester.
SiRNA molecules were synthesized, HPLC purified, deprotected and annealed by Dharmacon **(where from??)**
Antisense ODN and siRNA were dissolved to appropriate concentration in nuclease free water and stored at –80.

Reverse transcription and Real Time PCR reaction

Cells were harvested after 6 hours (ODN molecules) or after 48 hours (siRNA molecules), washed twice in PBS and RNA was isolated with Qiagen kit **(from??).** The obtained RNA was treated with RQ-DNAse and re-purified with Qiagen kit. The reverse transcription was performed in 20 μL total volume with 300 ng of RNA using M-MLV Reverse transcriptase (Invitrogen) and random hexamers at 10mM concentration. Then1uL of cDNA was used for the real time PCR reaction. The reaction was performed in triplicates each time for *bcl-6* and *18S* as a reference gene. The negative control was carried out on RNA (no RT reaction).
Primers and probe for the Real Time PCR were designed with Molecular Beacon Program from BioRad and synthesized (+HPLC purified) by Nucleic Acid Facility(University of Pennsylvania). The sequences were as follows:

1) for *bcl-6*:Forward primer: 5'- CCA ACC AAG CTG AGT GCCAG; Reverse primer: 5'- GGT GCA TGT AGA GTG GTG AGT G; probe: 5'-CTC CAC CAT CCC ACAAGC CAG CCG. The probe was labeled with FAM at 5'end and Black whole1 quencher at the 3' end;
2) for *18S*: Forward primer: 5'- GGA CAT CTA AGG GCA TCA CAG ACC-3'; Reverse primer: 5'- TGA CTC AAC ACG GGA AAC CTC AC-3'; probe: 5'- TGG CTG AAC GCC ACT TGT CCC TCT AA-3' (FAM at the 5' end and TAMRA quencher at the 3' end)- hybridizes between 1294 and 1614 in human 18S

The protocol for PCR reaction consisted of: 50°C for 2 min, and denaturation at 95°C for 10 min, followed by 36 cycles of 15 sec denaturation at 92°C and 1 min of annealing and elongation at 60°C. The expected 133bp *bcl-6* PCR product was resolved on 1% agarose gel to confirm the specificity of the reaction

Western blotting

Cells incubated for the appropriate time with nucleic acid molecules were washed and lysed in triple lysis buffer to obtain a total (nuclear and cytoplasmatic) protein cell extract. In each case equal amount (100μg) of the total protein was resolved on 10% polyacrylamide gel and transferred to the PVDF membrane and then probed with the primary antibody against bcl-6 (rabbit polyclonal, C-19) at 1:500 dilution from Santa Cruz Biotechnology (where???) at 4°C overnight. After 3x washing in tbs-t buffer the secondary antibody (anti-rabbit, HRP conjugated), 1:1000 dilution, was incubated with the membrane for 1 hour at room temperature. The visualisation was performed with ECL (+) reagent. The same PVDF membrane was then stripped with stripping buffer and probed with antibody against beta actin (1:3000 dilution).

Results

SQRM screening

Out of 19 tested SQRM we were able to identify one (1310) which hybridized well. This molecule (numbered by the nucleotide position in the mRNA according to the sequence from Gene Bank) yields a 10-fold increase in fluorescence compared to background signal **Fig1**.

RNaseH assay

To confirm the hybridization we carried out an in vitro RNaseH cleaving assay with one hybridizing (1310) and two of the non-hybridizing molecules (951, 1487). After incubation of in vitro transcribed bcl-6 mRNA (2,4kB) with RnaseH in the presence of different molecules we were able to visualize the expected fragments on agarose gel. Only the hybridizing molecule, 1310, gave the two fragments of expected size ~1,1kB and ~1,3kB. Bcl-6 mRNA incubated with non-hybridizing molecules and with enzyme only remained intact (~2,4kB). **Fig2.**

1310 molecule tested with RNA isolated from cell lines (fluorimetric assay)

We demonstrated the increase in fluorescence signal when 1310 molecule was incubated with total cellular RNA from bcl-6 expressing cell line and this effect was dose-dependent. We did not see any significant changes in fluorescence signal when the same molecule was incubated with RNA from K562 cells. **Fig3.**

Transfection efficiency

We have established a good and reliable delivery method for the Loucke cell line using the nucleoporation procedure. The efficiency of the delivery with fluorescein labeled ODNs was~95%, and cell viability over 90%. **Fig 4- you should have this picture**

Cell viability

The viability of cells followed for 4 consecutive days differed substantially depending on which molecule was used for transfection. In case of hybridizing molecule 1310 we saw the most dramatic drop in viability(from 100 to less then 50% within first 24 hours), which we did not see to this extend for other molecules or in control cells. We noticed some drop in cell viability for 1487 molecule, but to a much lesser extend then for 1310 (65%). **Fig 5**

Real Time PCR and western blot analysis

For cells transfected with antisense ODN we were able to demonstrate a 7-fold decrease in bcl-6 mRNA expression level for the 1310 molecule. We also noticed some 2,5 decrease for 1487 molecule, We did not see any significant differences in bcl-6 mRNA expression for other molecules and control cells. **Fig 6**

In case with siRNA molecules we have noticed that the 2241 molecule gave to most reduction in bcl-6 expression and this reduction was dose-dependent with saturation at 5μg **fold?** Fig. 7 and 8

The western blot analysis was consistent with Real Time PCR results and showed the greatest decrease in protein expression for the1310 molecule in case of antisense ODN, and for 2241 molecule in case of siRNA. **Fig 9 (a,b).**

Discussion

Much progress has been made in understanding the molecular pathogenesis of many diseases and many genes responsible for cellular transformation have been identified. We hypothesize that if the function of these genes were shown to be either completely or relatively tumor specific they would become legitimate targets for therapeutic manipulation of their expression. More effective, less toxic cancer treatments could reasonably be expected to result if the strategy were successful. In our efforts we have focused primarily on modulating gene expression on mRNA level. Destabilizing mRNA is a widely applied "antisense" strategy, using ribozymes, DNAzymes, antisenseRNA or DNA, or newly developing approach called post-transcriptional gene silencing. All these strategies are based on hybridization between given mRNA and complementary sequences of introduced or expressed NA molecule. We have developed a reliable mapping strategy, which allows us to predict the sites accessible for hybridization within target mRNA. Since our mapping experiments were carried out in a protein free system and at different ion concentration, then present in the intracellular environment, it was important to test the results in cellular systems. Different molecules, tested previously in vitro, were delivered into human Burkitt lymphoma cell line expressing bcl-6 (Louckes). The antisense sequence 1310, which was hybridizing best according to our mapping experiments, yielded the best results in the cells too. In our gene expression analysis based on the Real Time PCR technic we demonstrate a 7-fold decrease in bcl-6 expression when cells were transfected with 1310, 2,5 fold decrease in case of 1487 molecule and no significant decrease in case of other molecules. We followed also the protein expression in western blot and similarly- we saw a significant decrease in BCL-6 protein expression when cells were transfected with 1310 molecule and no significant decrease in case of all remaining molecules. These results correlated well with cell viability tested with trypan blue exclusion. When cells were transfected with 1310 molecule we saw a dramatic decrease of cell viability from 100 to less then 50%. We did see some decrease in cell viability in case of 1487 molecule, but to a lesser extend (~65%). Other molecules did not affect the cell viability significantly. One could argue that the results given by 1310 molecule could be not specific since this was the only one ODN containing G quartet in its structure. Therefore we have included in our experiments an additional, scrambled molecule (33-mer) with G quartet and the same base content like 1310. The results obtained with scrambled molecule were not giving either changes in cell viability, nor in bcl-6 expression levels.

According to our mapping experiments the sequence 1487 did not hybridize well with its bcl-6 target. To our surprise this molecule gave some decrease in cell viability, which correlated with some decrease of bcl-6 expression in our Real Time PCR assay **(I don't know western for sure)**. As mentioned above our mapping experiments were carried out in "cell like" environment, regarding pH and $MgCl_2$ ion concentration, but we did not include any cellular proteins, or other ions, which may interfere with RNA folding in vivo. We had to test 19

different molecules to find one hybridizing well. We hypothesize that our "hit" rate may be improved by making the in vitro conditions more physiological and probably we would be able to identify more molecules hybridizing well.

The question if bcl-6 could be a potential therapeutic target remains open. The reports from clinical analyses in lymphoma patients gave some confusing results. There are studies showing that over-expression of bcl-6 may be a good prognostic factor, but there are also studies showing the opposite (**see the introduction**). Recent findings from Pasqualucci et al. (Pasqualucci L, Blood, 2003) may help to investigate the role of bcl-6 in lymphoma patients. The report from this group shows clearly that there are some chromosomal translocations, which are specifically associated with DLBCL, but are not present in normal GC B cells. This type of mutations disrupts the negative autoregulation of bcl-6 gene in lymphoma, and may be creates the "identification mark" for the subset of lymphoma patients in which bcl-6 could be a potential therapeutic target.

We did see some interesting biology in our experiments on human lymphoma cell line over-expressing bcl-6. When cells were transfected with 1310 molecule, we observed a decrease in cell viability, which correlated well with bcl-6 mRNA and protein down-regulation. We did not see these changes in cells transfected with other molecules, where bcl-6 down-regulation was not achieved. If cell death was related to bcl-6 down-regulation, needs to be tested in further experiments where one would check the correlation with some of the down-stream targets of bcl-6. Still, we believe that bcl-6 remains an interesting therapeutic target, at least for a subset of lymphoma patients.

Furthermore we have compared the sequence accessibility for antisense ODN and siRNA approach. In our RNAi experiments we have focused on four sequences: 1310, 2241, 1487, 952. Despite to our expectation we saw bcl-6 mRNA and protein decrease in cells, which have been transfected with 2241 sequence. This sequence did not hybridize well in our mapping studies and did not diminish bcl-6 expression when used as antisense ODN. We speculate that particularly this sequence may become accessible for RNAi approach because of the activity of unwindase, an enzyme, which is incorporated into RISC complex, and which does not play a role in antisense mechanism. We can also not exclude that mapping in more physiological conditions would reveal 2241 molecule as hybridizing well- as discussed above. On the other hand the 1310 sequence, which was working best as antisense, did not work as siRNA at all. Our original 1310 sequence was a 33-mer. To design an siRNA molecule we had to shorten this sequence substantially to obtain a 21-mer. We have prevent mostly the central part of the molecule and "cut off" the ends. Since it is known that even moving the sequence by one nucleotide may convert the molecule from hybridizing well to a non-hybridizing, therefore we think that shortening the molecule by 11 nucleotides probably was the cause for loosing its properties.

Taking together we think that we have developed a good and reliable tool for identifying sequences accessible for hybridization within mRNA. Our *in vitro* results correlate well with studies on cell lines (*in vivo*). We speculate that we could improve our in vitro mapping results by carrying out experiments in more physiological conditions.

Tab.1 Sequences of SQRM used for testing bcl-6 mRNA. The sequences are numbered/named by nucleotide position in the mRNA. The palindromic fragments in each SQRM are marked in bold.

Sequence name	Nucleotide sequence
23	5'-TTC TTA GAA GTA GTG ATG CAA GAA
27	5'-TAG AAG TGG TGA TGC AAG AAG TTT CTA
638	5'-CTG GGG GCA AAG GCT CTG CTC TCA CAC CCA G
736	5'-CTC CTC TTC TCC GAT GAG GAG
804	5'-`GGC ACT CCC ATG TGA TAG TGC C
951	5'-GGC TGA GGG CCT CAA ACC TGC TGC CCC CTC AGC C
952	5'-GCT GAG GGC CTC AAA CCT GCT GCC CCC TCA GC
953	5'-CTG AGG GCC TCA AAC CTG CTG CCC CCT CAG
954	5'-TGA GGG GGC AGC AGG TTT GAG GCC CTC A
1061	5'-TGC ATT TCG AGC CCC CCA ATG CA
1159	5'-GCC TGG AGG ATG CAG GCA TTC TTA CTG CTG CAG GC
1310	5'-AGG CTC GTG GGG AAA GGC GGC CCA GCT CAG CCT
1486	5'-GGC TCT CCC CGC AGC AGC AGC GAG AGC C
1487	5'-GCT CTC CCC GCA GCA GCA GCG AGA GC
1924	5'-TAC AAA TGC GAA ACC TGC GGA GCC AGA TTT GTA
1936	5'-ACC TGT ACA AAT CTG GCT CCG CAG GT
1941	5'-CGG AGG TGG GCC ACC TGT ACA AAT CTG GCT CCG
2241	5'-AAG CAT GGA GTG TTG ATG CTT
2246	5'-TGG AGT GTT GAT GCT TTC GTC TCC A
Scramble	

Tab.2 sequences of siRNA molecules

Sequence name	Nucleotide sequence (sense strand)
952	5'-GGG GGC AGC AGG UUU GAG GdTdT
1310	5'-AGC UGG GCC GCC UUU CCC CdTdT
1487	5'-CUC GCU GCU GCU GCG GGG AdTdT
2241	5'- GCA UCA ACA CUC CAU GCU UdTdT